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Detection of *Wolbachia* Endosymbiont in Several Agriculturally Important Insect Parasitoids in Bogor, Indonesia

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ABSTRACT

Wolbachia that have been found to be maternally inherited in arthropods, including insects behave primarily as a reproductive parasite by inducing feminization and/or death among genetic males; causing cytoplasmic incompatibility, and activating parthenogenesis, in host insects. This research aims to detect the presence of *Wolbachia* in several parasitic insects and to determine the diversity of *Wolbachia* at the supergroup level. Insect genetic samples were collected, amplified using *wsp* (*Wolbachia* surface protein) gene and with the sequences then analyzed using species homologues drawn from the Genbank database. These activities were conducted to detect the intra-cellular presence of *Wolbachia* and assembled the phylogenetic tree of the respected bacteria. Phylogenetic analysis confirmed that *Wolbachia* existed in the samples belonging to the supergroup A (found in *Cherapron* sp.) and supergroup B (found in *E. japonica*). *Wolbachia* were detected in seven out of nine wasps i.e. *Anagyrus lopezi*, *Ceraphron* sp., *Microplitis manilae*, *Brachymeria lasus*, *Scelionidae* sp01, *Trichogramma* sp, and *Exorista japonica*. Single infection by *Wolbachia* supergroup A was detected in *A. lopezi*, *Ceraphron* sp., *M. manilae*, and *Scelionidae* sp01. Meanwhile single infection by *Wolbachia* supergroup B was detected only in *E. japonica*. Double infection by both supergroups occurred in *B. lasus* and *Trichogramma* sp. samples.

INTRODUCTION

The interaction between insects and bacteria have evolved over millions of years, usually forming a symbiotic association that ranges from mutualism or commensalism, to parasitism. These endosymbionts provided either an obligate or facultative function. Obligate endosymbionts were maternally inherited and generally provided a nutritional benefit, supplying essential nutrients, and specialized to specific host organs (Baumann, 2005). Removal of this endosymbiont resulted in notably agile body weight and meager growth

rate and the effects were persisted to the offspring (Kuriwada et al., 2010). In comparison, facultative endosymbionts were not crucial for host continuity and their existence could be neutral, beneficial or even detrimental to the host (Oliver, Degnan, Burke, & Moran, 2010). Facultative endosymbionts living in the haemolymph of the insect, in some cases, enabled to horizontally transfer between hosts (Ishii, Matsuura, Kakizawa, Nikoh, & Fukatsua, 2013). One common example is *Wolbachia* which is the main topic of this study. *Wolbachia* comprises a group of bacterial endosymbiont that belongs to the order of

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Rickettsiales, family Anasplamataceae. *Wolbachia pipientis* was the only species member of genus *Wolbachia* (Dumler et al., 2001). *Wolbachia* is also an intracellular bacteria found in arthropods and nematodes. *Wolbachia* was also reported to infect for about 70 % insect species, and a large number of other arthropods (including mites, scorpions, spiders, springtails, and terrestrial isopods), as well as filarial nematodes (Werren, Windsor, & Guo, 1995). *Wolbachia* multiplies through binary fission within host cell vacuoles where the bacteria are protectively enveloped by host origin's membrane. In arthropods, *Wolbachia* was mostly found in the bacterial cells' cytoplasm of the reproductive organs, and also detected in other tissues, including hemocytes and nerve tissues (Louis & Nigro, 1989). In arthropods and nematodes, *Wolbachia* was inherited to the host offspring via transovarial transmission (Werren, 1997).

Wolbachia infections, in arthropods, were frequently associated with conditions and changes disruptive to host reproduction, such as feminization and/or death among genetic males; cytoplasmic incompatibility, and activation of parthenogenesis (Stouthamer, Breeuwer, & Hurst, 1999). The endosymbiont enabled to manipulate their insect hosts' reproduction system through suppressing the insect pests or inducing biocontrol agent population and their function. Such mechanism indicated the ability of the parasitoids as biocontrol agent. The effect of *Wolbachia* as wasps parasitoid has not been thoroughly investigated. In some insect species, *Wolbachia* (Hurst & Jiggins, 2000), including several economically important parasitoids like *Asobara japonica* (Dedeine, Boulétreau, & Vavre, 2005), *Trichogramma brassicae* (Rahimi-Kaldehy, Ashouri, & Bandani, 2018) and *Cotesia sesamiae* (Mochiah, Ngi-Song, Overholt, & Stouthamer, 2002) were actually necessary for their host reproduction. The females of genus *Asobara* had to be infected with *Wolbachia* to produce oocytes (Dedeine, Boulétreau, & Vavre, 2005). However, although required, the infection might reduce reproductive effectiveness. *Wolbachia*-infected *A. japonica* females usually produced low quantity of male progeny, and obligate *Wolbachia*'s infections in *A. japonica* could also result in parthenogenesis. In addition, infected population reproduced by sexually and parthenogenesis, while uninfected only sexually (Reumer, van Alphen, & Kraaijeveld,

2012). These facts indicated that the infection by *Wolbachia* can affect the population demographics of insect hosts (Xi, Khoo, & Dobson, 2005).

The information about *Wolbachia* infection on beneficial and/or parasitic insects is still limited in Indonesia. The leading topic for examination in the redistribution of these parasitoids as biocontrol agents is whether the selected strains are compatible with native or not. *Wolbachia*-specific *wsp* gene in this study was taken from several species of parasitic insects, including hyperparasitoids. The genes were amplified using PCR and the sequences were analyzed. The information obtained is critical to determine the infection status of parasitic insects. The current initiative detection of *Wolbachia* infection in parasitoids would ensure the successful implementation of *Wolbachia* in both basic and applied researches on the biocontrol of insect pests.

MATERIALS AND METHODS

Insect Collection

The collected *Wolbachia* species, their pest host as well location and date of samples taken were presented in Table 1. When collecting insect samples the insects were selected in size that fit with 0.1 ml in Eppendorf tubes. If this size was exceeded, only the abdomen parts were taken and extracted. The collected were stored in a freezer at -20 °C for preservation and to halt bacterial endosymbiont metabolism processes inside the insect.

DNA Extraction

DNA was obtained by pulverizing the insect tissues taken from the reproductive organs, abdomen, or the entire body depending on the size of insect samples. For each insect tissue samples, a total of 200 µl CTAB buffer was added to an Eppendorf tube, and then, the insect tissue was inserted. Insect samples were macerated within the tube using a plastic micro pestle until small bits of the chitinous exoskeleton was remained, and then incubated at 65 °C for 15 minutes. A total of 200 µl chloroform-isoamyl alcohol 24:1 (v/v) was added to the tube and inverted for 30 minutes. The tubes were centrifuged at 10⁴ rpm (25 °C) for 20 minutes to produce supernatant. The supernatant was placed into another Eppendorf tube with the volume of 150 µl. RNase with the volume of 2 µl was added into the tube containing supernatant and incubated at 37 °C for 1 hour. A total of 15.2 µl NaOAc 3 M was

put into the tube and followed by the addition of 480 µl absolute ethanol, then incubated in a refrigerator at a -20 °C for 3 hours. The tube was centrifuged at 12,000 rpm (4 °C) for 15 minutes. The supernatant produced from these step was removed. The pellet containing DNA samples was then washed with 800 µl of 70 % ethanol and centrifuged at 12,000 rpm (4 °C) for 3 minutes. The remaining supernatant was removed and the pellet were suspended with 50 µl TE buffer.

Amplification of *Wolbachia* Surface Protein (*wsp*) Gene

Amplification of the *wsp* gene for the presence of *Wolbachia* was performed using specific primers to produce 632 base pairs fragments. The general *wsp* primers used in the assay were 81F (5'TGG TCC AAT AAG TGA TGA AGA AAC 3') and 691R (5'AAA TAA ACG AAT CA 3') (Zhou, Rousset, & O'Neill, 1998). The PCR amplification reactions were carried out in a thermo cycler (Gene Amp PCR System 9700, PE Applied Biosystems, USA) using 25 µl reaction mixtures consisting of 1.25 µl of 20 pmol primers, 8 µl Dream Taq PCR Master Mix 2X, 13.5 µl ddH₂O, and 1 µl the crude DNA extract. The amplification was conducted with initial denaturation at 94 °C for 2 minutes, then trailed by 30 cycles comprising of 94 °C for 30 seconds, 50.7 °C for 1 minute and 72 °C for 1 minute, and a final extension for 4 minutes at 72 °C (Pourali, Roayaei Ardakani, Jolodar, & Razi Jalali, 2009). The samples were then tested for the classification super group A or B. The method was following Zhou, Rousset, & O'Neill (1998). Primers pairs 136 F (5' TG AAA TTT TAC CTC TTT TC 3') and 691R (5'AAA AAT TAA ACG CA 3') which would amplify

the 556 bp fragment and the positive reaction would bring the samples into *Wolbachia* supergroup A. While, the primer pair 81F (5'TGG TCC AAT AAG TGA TGA AGA AAC 3') and 522R (5' ACC AGC TTT TGC TTG ATA 3') would amplify the 442 bp fragment found in *Wolbachia* of supergroup B.

Phylogenetic Analysis

Aligning two representative *wsp* gene sequences in this research utilized Muscle on a MEGA 6.1 software. The sequencing process was conducted offsite by PT. Genetika Science, Jakarta. Sequences database downloaded from Genbank (<https://www.ncbi.nlm.nih.gov/genbank/>) that recorded all present known supergroups of *Wolbachia* were admitted in the analysis. Phylogenetic analyses were conducted using the Neighbor-Joining calculation, with probabilities instigating 1000 bootstrap repeats. The sequences achieved in this study were then deposited in the DNA Data Bank of Japan (DDBJ) under the accession numbers LC361452, LC361453.

RESULTS AND DISCUSSION

Screening for *Wolbachia* Infection in Several Parasitic Insects

The PCR amplification of nine insects wasps DNA using *wsp* specific primer for the existence of *Wolbachia* was presented in Fig. 1. The *wsp* gene was considered to be an accurate marker for the presence of bacterial endosymbiont *Wolbachia*, due to great activity in the gene making it likely to obtain a proper outcome while utilizing data for phylogenetic analysis (Vavre, Fleury, Lepetit, Fouillet, & Boulétreau, 1999).

Table 1. *Wolbachia* infection from several parasitic insects and their locality

Species	Host	Locality	Year of Collection	<i>Wolbachia</i> infection
<i>Apanteles taragamae</i>	<i>Diaphania indica</i>	Cihideung	2016	-
<i>Microplitis manilae</i>	<i>Spodoptera litura</i>	Situgede	2016	A
<i>Stictopisthus</i> sp.	<i>Diaphania indica</i>	Petir	2016	-
<i>Anagyrus lopezi</i>	<i>Phenacoccus manihoti</i>	Dramaga	2016	A
<i>Ceraphron</i> sp.	<i>Apanteles taragamae</i>	Bantarjati	2016	A
<i>Exorista japonica</i>	<i>Mythimna separata</i>	Tsukuba	2016	B
<i>Brachymeria lasus</i>	<i>Erionota thrax</i>	Situgede	2013	A, B
<i>Scelionidae</i> sp01.	Unidentified	Cikabayan	2013	A
<i>Trichogramma</i> sp.	<i>Corcyra cephalonica</i>	Babakan	2013	A, B

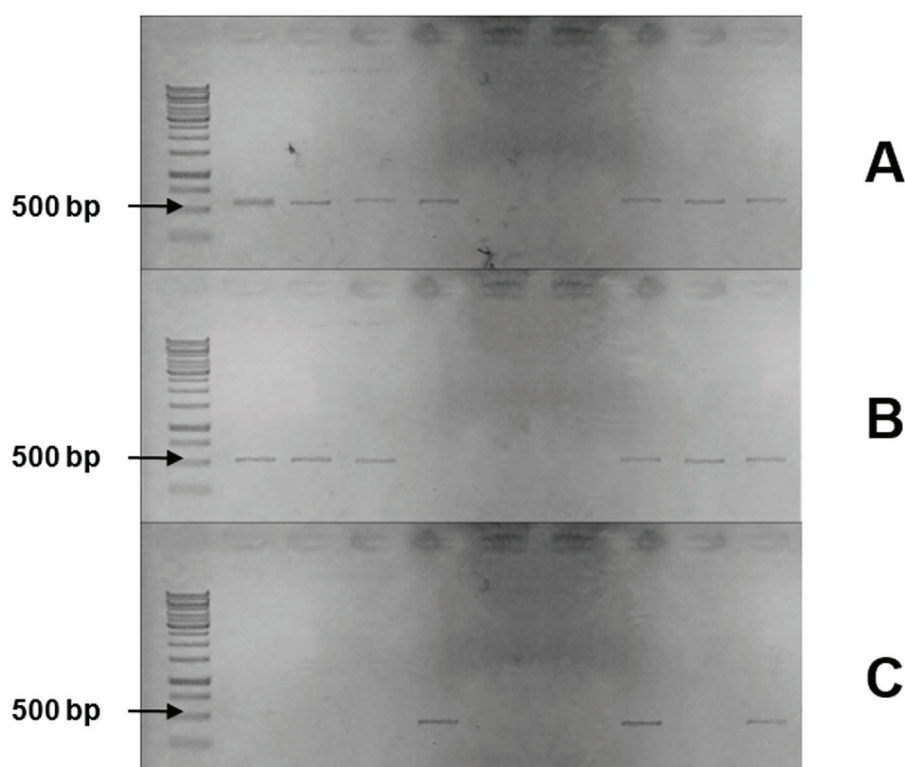


Fig. 1. PCR amplification of *Wolbachia* infection in several parasitic insects. Lane M: 1 kbp DNA marker ladder, 1: *M. manilae*, 2: *A. lopezi*, 3: *Ceraphron* sp., 4: *E. japonica*, 5: *A. taragamae*, 6: *Stictopisthus* sp., 7: *Brachymeria* sp., 8: *Scelionidae* sp.01, 9: *Trichogramma* sp. A: general *wsp* gene amplification of *Wolbachia*, B: *wsp* supergroup A gene amplification of *Wolbachia*, and C: *wsp* supergroup B gene amplification of *Wolbachia*.

The insect samples were collected in seven locations in the Bogor area, with the exception of one sample obtained from Japan International Research Center for Agricultural Sciences (JIRCAS). The results showed that PCR amplification of sampled tissues detected the presence of the general *wsp* primer in seven out of the nine analysed-parasitic wasps. These 7 *Wolbachia*-positive samples were then amplified using *wsp* A and *wsp* B primers to determine the supergroup type for each *Wolbachia* infection. *Wolbachia* supergroup A was detected in *M. manilae*, *A. lopezi*, *Ceraphron* sp., and *Scelionidae* sp.1 whereas, *Wolbachia* supergroup B was detected only in the *E. japonica* sample. Meanwhile samples from *B. lasus* and *Trichogramma* sp. were infected by both *Wolbachia* supergroup A and B (Table 1). Double infection by *Wolbachia* naturally occurs in some insect species. An additional survey found that *Wolbachia* infected 35 % of neotropical insects from both supergroup A and B (Werren, Zhang, & Guo, 1995). Referenced studies reported evidence of

multiple *Wolbachia* infections, among: three species of *Nasonia*; yellow *Eurema hecabe* butterflies; raspberry beetle *Byturus tomentosus*; and other insects (Malloch, Fenton, & Butcher, 2000; Narita, Nomura, & Kageyama, 2007).

Among the 7 positive infected insect species, 5 of them were agriculturally beneficial parasitic wasps and used for pest control. While, the other two were categorized as hyperparasitoids (*Ceraphron* sp. and *Scelionidae* sp.01). *A. lopezi* was introduced to Indonesia from Thailand in 2014 to control cassava mealybug *Phenacoccus manihoti*. These insect pest was considered invasive and might cause significant damages to the plant and yield losses up to 30-40 % (Wyckhuys, 2014). *M. manilae* was known as a polyphagous parasitoid that attacks armyworm *Spodoptera litura*, the most important insect pest of many agricultural crops in Indonesia (Shepard, Barrion, & Litsinger, 1987). *B. lasus* was a gregarious endoparasitoid predator of the banana leaf roller *Erionota thrax* (Erniawati & Ubaidillah, 2011).

The polyphagous egg parasitoid *Trichogramma* is one of around 80 genera from the family Trichogrammatidae, that has been most used as biocontrol agents in agricultural crops (Cônoli & Grenier, 2009). *Exorista japonica* was often found on larval stages including noctuid larvae of a typical armyworm *Mythimna separata*, typical cutworm *Spodoptera litura*, and the cabbage armyworm *Mamestra brassicae*. In choice test experiment, *E. japonica* parasitized other Lepidopteran species such as *Bombyx mori*, *Spilarctia subcarnea*, and *Hyphantria cunea* (Yang, Hui, ShuYan, Zhen, & XuGen, 2012).

This experiment found also confirmed the first evidence of *Wolbachia* infection on *A. lopezi*, *M. manilae*, and *B. lasus*. While other reports have indicated the *Wolbachia* infection on at least 14 species of *Trichogramma* (Schilthuizen & Stouthamer, 1997). Although the bacteria has been detected in several parasitic wasps, the mode of preferences of *Wolbachia* to a certain phenotype is still unclear. Weeks & Breeuwer (2001) reported that generally, *Wolbachia* infection in *Trichogramma* species would induce parthenogenesis. The induced-parthenogenesis female produced only daughters from both their fertilized and unfertilized eggs. These resulted in the increase of female offspring yet, lack of males within the population. These condition was expected to reduce number of subsequent generations due to the lower mating incidences. The lower number of generations was then considered beneficial for pest control efforts.

Wolbachia infected broad kind of insects and played vital roles in host reproduction. *Wolbachia*, cytoplasmic incompatibility (CI), feminization and death of males; and thelytokous parthenogenesis (Stouthamer, Breeuwer, & Hurst, 1999). CI firstly observed and described in *Culex pipiens*. Other researchers also reported the phenomenon in the of *Drosophila* sp. and parasitic wasps *Nasonia* spp. (Werren, 1997). CI caused by the endosymbiont infection resulted in isolation of reproductive function between the population. The bacteria were located within internal sperm structures during the stage of spermatogenesis, although they have also been seen in sperm before spermatogenesis. CI would be induced when the mating was happened between infected male with sterile female or a female carrying different strain of *Wolbachia* (Rostami, Madadi, Abbasipour, & Sivaramakrishnan, 2016). In unidirectional CI

crosses, paternal chromatin neglected to gather appropriately in the main cell cycle and was lined on the metaphase plate amid the first mitosis, thus causing failure in the process of embryo development (Beckmann, Ronau, & Hochstrasser, 2017). This phenomenon has caught the attention of researchers, especially those interested in the use of parasitoids as biocontrol agents. In biological control of pest, the number and proportion of female (as opposed to male) parasitoids is an important factor in effectively regulating pests. CI could be likely destructive to control attempts, as it resulted in lower female populations and fecundity. On the other hands, it also informed the phenotypes of parasitic wasps that was mostly to CI when infected by *Wolbachia*. CI also could serve as a basis of evaluation on mating compatibility between *Wolbachia*-infected populations and non-infected populations or infected population by a different strain of *Wolbachia*. On *Habrobracon hebetor* wasp, the infection of *Wolbachia* affected the host mating preference and the transmission to the net progenies (Bagheri, Talebi, Asgari, & Mehrabadi, 2019). To determine which parasitoid should be selected for redistribution as an effective biocontrol agents, it is critical to consider whether the introduced parasitoids would be compatible for reproduction with the native population, with regard to the strain of *Wolbachia* each population hosts. The crucial point that must be considered is the infection status of each biocontrol agent that will be released in the field. The release of infected insects into a territory that has an uninfected native population is similar with the technique of insect sterile which could threaten the success of the biocontrol agents due to CI induction. Mochiah, Ngi-Song, Overholt, & Stouthamer (2002) reported that mixing uninfected populations of *Cotesia sesamiae* with infected populations that revealed CI, led to a transient, but possibly long-term, reduction in population growth rate. Authors confirm that screening for *Wolbachia* infection should be conducted to all biocontrol agents. The evaluation should also be tested for fitness costs associated with prevalence that could be likely destructive to biological control program. Many reports have affirmed that reproductive incompatibility problems could and should be avoided by assessing the infection status of the endemic population and those of the introduced population, and making appropriate adjustments.

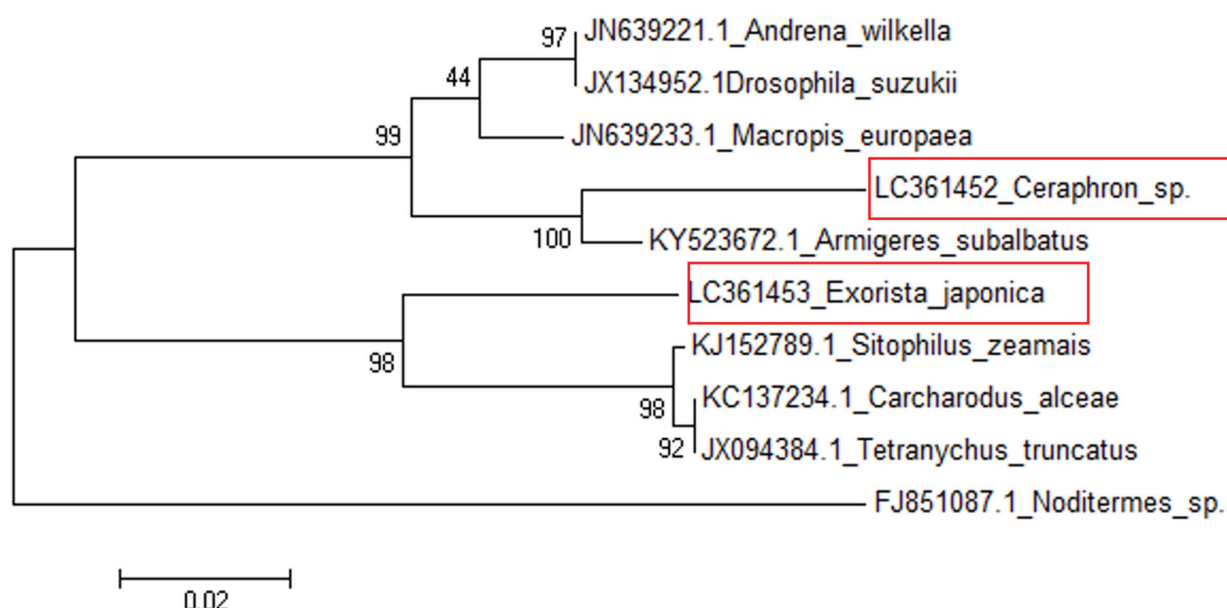


Fig. 2. Neighbour-Joining tree based on *wsp* gene sequences. Number above branches are bootstrap values (1000 replications). Sequence obtained from this study have been submitted in the DDBJ (with red box) meanwhile others sequences were downloaded from Genbank. All sequences were written down as accession number then followed by host species of *Wolbachia*.

Phylogenetic Analysis

To confirm PCR products, a gene fragment from samples of each representative insect was sequenced. Homology searches with BLAST (<https://www.ncbi.nlm.nih.gov/BLAST/>) revealed that *Ceraphron* sp. sample's sequence at the first rate hit to the *Wolbachia*'s *wsp* gene in *Drosophila incompita* (Acc No. CP011148.1) with 96 % identity. Meanwhile, the *E. japonica* sample was the most similar to the *wsp* gene of *Wolbachia* in *Polygonia caureum* (Acc No. AB094378.1) with 99 % identity. Eight *wsp* sequences of *Wolbachia* downloaded to serve as the dataset for assembly of phylogenetic analysis merged the *wsp* sequence derived from the samples of *Ceraphron* sp. and of *E. japonica*. The phylogenetic analysis illustrated a significant distinctive between *Wolbachia* supergroups and the most frequent *Wolbachia* endosymbionts were grouped into supergroup A and B. The topology of the observed *Wolbachia* also confirmed that *Wolbachia* sample from *Ceraphron* sp. was infected by supergroup A and *E. japonica* was infected by supergroup B (Fig. 2).

CONCLUSION AND SUGGESTION

Wolbachia as a bacterial endosymbiont has been detected in several parasitic insects i.e.

A. lopezi, *Ceraphron* sp., *M. manilae*, *B. lasus*, *Scelionidae* sp01, *Trichogramma* sp., and *E. japonica*. Using PCR amplification and analysis, a single infection by *Wolbachia* supergroup A was detected in *A. lopezi*, *Ceraphron* sp., *M. manilae*, and *Scelionidae* sp.1; while *E. japonica* was infected by *Wolbachia* supergroup B. Double infection by both supergroups occurred in *B. lasus* and *Trichogramma* sp. Two representative samples from this study have been sequenced and submitted in the DDBJ under accession number: LC361452 and LC361453. Further researches are still needed in investigating the effect of *Wolbachia* infection on the parasitic insects to support the more environmental friendly and safer biocontrol program.

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